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Improvement of Na^+-K^+ ATPase Activity and Gene Expression Associated with Glomerular Ultrastructural Protection in Diabetic Nephropathy by *Acacia senegal*: Role of Oxidative Stress Disturbances

Mejora de la actividad Na^+-K^+ ATPasa y la Expresión Génica Asociada con la Protección Ultraestructural Glomerular en la Nefropatía Diabética por *Acacia senegal*: Papel de las Alteraciones del Estrés Oxidativo

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SUMMARY: This study assessed the effects of Acacia Senegal (AS) combined with insulin on Na^+/K^+-ATPase (NKA) activity and mRNA expression, serum glucose, renal function, and oxidative stress in a rat model of diabetic nephropathy (DN). Sixty rats were equally divided into six groups: normal control, normal+AS, diabetic (DM), DM+insulin, DM+AS, and DM+insulin+AS groups. Diabetes mellitus (type 1) was induced by a single injection of streptozotocin (65 mg/kg), and insulin and AS treatments were carried until rats were culled at the end of week 12. Serum glucose and creatinine levels, hemoglobin A1c (HbA1c) were measured. Renal homogenate levels of NKA activity and gene expression, malondialdehyde, superoxide dismutase (SOD), catalase and reduced glutathione (GSH) were evaluated as well as kidney tissue histology and ultrastructure. Diabetes caused glomerular damage and modulation of blood and tissue levels of creatinine, glucose, HbA1c, malondialdehyde, NKA activity and gene expression, SOD, catalase and GSH, which were significantly ($p<0.05$) treated with AS, insulin, and insulin plus AS. However, AS+insulin treatments were more effective. In conclusion, combined administration of AS with insulin to rats with DN decreased NKA activity and gene expression as well as oxidative stress, and improved glycemic state and renal structure and function.

KEY WORDS: Na^+-K^+ ATPase; *Acacia senegal*; Diabetic nephropathy; Oxidative stress; Glycemic control.

INTRODUCTION

Diabetic nephropathy (D.N.) is a potentially life-threatening microvascular complication of diabetes mellitus (D.M.), which leads to renal failure if not managed. It has been estimated that up to forty percent of diabetes patients complain of D.N. (Gheith *et al*., 2016). In diabetes, chronic hyperglycemia leads to non-enzymatic glycosylation reactions and advanced glycosylation end products (AGEs). It was found that AGE deposition causes kidney structural changes, including glomerular basement membrane thickening, hypertrophy, and glomerulosclerosis (Li *et al*., 2018). The most common symptoms of D.N. include severe albuminuria (more than 300 g/day), hypertension and irreversible decline in glomerular filtration rate (GFR).

Acacia senegal (AS) is a dried exudate acquired from *Acacia senegal* and *Acacia seyal*. AS is considered as a safe dietary fibre since no adverse effects were reported after AS use, even at very high doses (Phillips *et al*., 2008). Over the last two decades, AS has been considered an inert substance. However, recently multiple actions of AS, such as antioxidant, lipid-lowering, and anti-inflammatory effects, have been reported (Ali *et al*., 2013). In some Middle Eastern countries, it treats chronic kidney and end-stage renal disease. Evidence shows that AS decreases blood pressure and plasma cholesterol levels in rats, and shows antimicrobial properties (Nasir, 2013).

Renal Na^+/K^+-ATPase (NKA) is an active transporter that maintains electrolyte homeostasis hence playing an

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essential role in transporting specific solutes. The defect NKA reduces glomerular filtration (G.F.) and tubular sodium reabsorption (Know *et al*., 2000). The NKA is essential for enzyme action and renal tubular sodium reabsorption (Codina & DuBose Jr., 2006). In the proximal tubular cells, sodium is reabsorbed actively by the luminal Na^+–H^+ exchanger and the Na^+–HCO_3^- co-transporter and extruded through the basolateral NKA. Moreover, NKA is a crucial component responsible for sodium reabsorption by the thick ascending part of the loop of Henle and distal convoluted tubule (DCT). In diabetes, proximal tubule NKA activity increases, enhancing sodium retention (Kim *et al*., 1998). A dysfunctional NKA is another characteristic of diabetes-sensitive tissues. A direct alteration of NKA function may also be caused by hyperglycemia-mediated oxidative stress. The altered activity of renal NKA in D.M. is only partially recovered by insulin treatment. (Nordquist *et al*., 2010).

To the best of our knowledge, no preceding report examined the effects of AS on renal NKA α-1 expression and NKA activity in rats with D.N. In addition, impairment in NKA activity and expression contributed to the pathogenesis and progression of D.N. (Vague *et al*., 2004). The results of affection of NKA activity and expression are contradictory. While It had been stated augmented enzyme activity and expression (Khadouri *et al*., 1987), many studies reported the opposite. They have shown reduced activity and expression of various units of this enzyme in different tissues, particularly RBCs, sciatic nerve, kidney, and heart (Raccah *et al*., 1998). Thus, we aimed to investigate the effects of AS, with or without insulin, on the expression and activity of renal NKA in rats with D.N. Several metabolic parameters were also assessed in these rats, including serum glucose, HBA1c levels, renal structure and function, and renal antioxidant elements.

MATERIAL AND METHOD

Study design. The present animal study used a randomized controlled design for twelve weeks.

Animals. Sixty male Sprague-Dawley rats aged 10 to 12 weeks were acquired from King Khalid University's animal house and kept there for this study. A standard rat chow was provided to the animals. According to the animal group, they were maintained at room temperature with a 12:12-hour light/dark cycle, and were given free access to water or water containing AS 10 %w/v. The King Khalid University's local committee for animal care approved all experimental procedures.

Animal groups. Six groups of ten rats were randomly selected. Group I (control group) rats were administered a vehicle (freshly prepared citrate buffer) intraperitoneally (I.P.) The second group (C+AS) consisted of normal rats given citrate buffer (once, intraperitoneally) and AS in drinking water (10 %w/v). In Group 3 (D.M.), rats were injected with streptozotocin (STZ) in a dose of 6.5 mg/100g intraperitoneally, once in a citrate buffer (0.1 M, pH 4.5) freshly prepared. Blood glucose was determined using retro-orbital blood samples to confirm the diagnosis of diabetes mellitus. Generally, 48 h post STZ injection, blood glucose was determined (non-fasting) and rats that had their blood sugar more than 20 mmol/L were considered diabetic. Diabetic rats in Group 4 (DM+Ins) were given insulin (1.6 IU/100g) single daily dose at 5:00 pm. The diabetic rats in Group 5 (DM+AS) received AS only in drinking water (10%w/v) for 12 weeks. The diabetic rats in Group 6 (DM+Ins+AS) were given insulin and AS at the same doses and routes as rats in (DM+Ins) and (DM+AS), respectively. All treatments for the animals in this study were given for 12 weeks (the whole time of the experiment).

Chemicals

***Acacia senegal*.** AS powder was obtained from Dar Savanna, Sudan (http://www.darsavanna.net). We prepared a 10% (w/v) AS solution by dissolving 100 grams of AS in 1000 mL of tap water Rats were provided with this AS solution in a glass bottle as a source of drinking water. A fresh solution is provided every 48 h. According to the study of Ali *et al*. (2013), the dose corresponded to approximately 2 g per 100g of body weight per day was administrated.

Insulin. A human insulin suspension was used. Diabetic rats in groups (DM+Ins) and (DM+Ins+AS) received insulin (1.6 IU/100g) in a single daily dose (subcutaneously) at 17:00 (Haughton *et al*., 1999).

Procedures

Collection of blood samples. Heparinized capillary tubes collected the retro-orbital blood samples under general anesthesia. Serum was prepared and labeled to determine glucose, sodium, potassium, creatinine, and urea concentrations. Prepared plasma (EDTA anticoagulant) was used to determine the glycosylated hemoglobin % (HbA1c). All the samples were stored at -80 °C until they were subjected to further biochemical analysis.

Collection of 24-h urine. Rats were held in metabolic cages for one day at the end of the experiment. Twenty-four hours urine proteins were assessed using the kit provided by Chondrex Inc, USA (Cat. No. 9040).

Tissue sampling. Rats were decapitated under diethyl ether anesthesia at the end of the study, and A quick nephrectomy

was done after their dissection. Harvested kidneys were used to prepared kidney homogenates (ultrasonic homogenizer, Omni International, Gainesville, VA), which were kept at -80°C until for determination of tissue antioxidant parameters, RNA isolation and real-time PCR, as well as tissue histology and transmission electron microscopy.

Biochemical analysis

Assessment of serum glucose and HbA1c. The glucose oxidase-peroxidase method was used to quantify serum glucose using the specific kit (SPINREACT), Spain (Cat. No. 1001190). The hemoglobin A1c (HbA1c) was determined using the specific kit (Crystal Chem INC), Spain (Cat. No. 80300).

Assessment of serum Na^+, K^+, and urea. Serum Na+ was determined colorimetrically by a commercial kit (Cat. No. KA4544, Abnova, Taiwan). Serum K^+ levels were determined using a turbidimetric kit (Elabscience, USA, Cat. No. E-BC-K279-M). Serum urea was assessed using the specific kit (Abcam, USA) (Cat. No. ab83362).

Assessment of serum and 24 h urinary creatinine. Creatinine was evaluated in blood and urine using creatinine specific kit (Abcam, USA) (Cat. No. ab65340)

Calculation of creatinine clearance. The specific scientific equation calculated creatinine clearance (ml/min).

Assessment of GSH, SOD, CAT, and MDA in renal homogenate. SOD (Cat. No.706002, Cayman Chemical. Michigan, USA), CAT (Cat. No.707002, Cayman Chemical. Michigan, USA), GSH (Cat. No.703002, Cayman Chemical. Michigan, USA), and MDA (Cat. No.10009055, Cayman Chemical. Michigan, USA) were measured in renal homogenates according to the instructions given by the manufacturer's.

NKA assay. The NKA activity was determined (in nmol of Pi released/mg of protein/min) (Linardi *et al*., 2011). A portion of left kidney tissue was homogenized in a solution containing 0.05 M Tris–HCl, pH 7.4, 0.25 M sucrose, and one mM EDTA. Following centrifugation (10 min, 1000g, 4 °C), We collected the supernatant and put it on ice. We homogenized and centrifuged the pellet twice, resuspended it in half its original volume, and homogenized twice more. The final pellets from the incubation were discarded, and the pooled supernatants were centrifuged for 15 min at 10,000 *g*. After the new supernatant was discarded, the pellets were resuspended in 2 mL of the same homogenized solution. For enzymatic activity detection, the mixture was stored at 80°C after aliquoting (Linardi *et al*. (2011). The protein concentrations were estimated using the method described by Bradford (1976).

NKA α-1 subunit quantitative real-time PCR (qPCR). qPCR was performed on kidney tissues harvested from all rats. Briefly, total RNA was isolated using TriFast reagent, triazole, and chloroform (PeqLab. Biotechnologie GmbH, Carl-Thiersch St. 2B 91052 Erlongen, Germany). RNA then reverse-transcribed using the QIAGEN Long Range 2 Step RT-PCR Kit. The cDNA was stored at - 85° C before being used for quantitative real-time RT-PCR against the housekeeping control gene, β-actin. The real-time PCR was done using SYBR Green reagent (Applied Biosystems, USA). The sequences of the primers used were: NKA α-1 subunit, Forward:

5'-CAGTGTTTCAGGCTAACCAAGAAA-3'; Reverse:5'-CGCCGACTCGGAAGCAT-3' (Linardi *et al*., 2011). β-actin, forward: 5'-GTCGTACCACTGGCATTGTG-3'; reverse: 5'-CTCTCAGCTGTGGTGGTGAA-3' (Wang *et al*., 2008). Relative gene expression levels was calculated using the comparative Ct technique.

Histopathological examination of the kidney. A small segment of the left kidney was processed for paraffin sectioning after being fixed in 10 % buffered formalin. Under a light microscope, 5 μm broad sections were stained with hematoxylin and eosin (H&E) and were investigated.

Transmission electron microscopy examination (TEM). The procedure involved the removal of the right kidneys, followed by the isolation of the renal cortex, which was subsequently cut into small pieces. These tissue samples were promptly immersed in a 2.5 % glutaraldehyde solution for 24 h and then rinsed with a phosphate buffer (0.1 M, pH 7.4). Post-fixation was carried out using a 1% osmium tetroxide solution buffered to pH 7.4 with a 0.1 M phosphate buffer at 4 °C for 1-2 h. After this step, the samples underwent further washing in phosphate buffer to eliminate excess fixative. Subsequently, they were dehydrated through a series of increasing ethanol concentrations and cleared using propylene oxide. The prepared specimens were embedded in Araldite 502 to create gelatin capsules, and polymerization was achieved by placing the capsules at 60 °C. Semi-thin sections, approximately 1 mm thick, were stained with toluidine blue to aid in orientation and observation. Ultra-thin sections, measuring 100 nm, were generated using an ultra-microtome and placed onto uncoated copper grids. Following double staining with uranyl acetate and lead citrate, three to five random micrographs were captured for each section using a JEM-1011-JEOL transmission electron microscope from Japan, operated at 80 kV.

Statistical analysis. Data were expressed as mean + standard error (SEM). SPSS version 22 (IBM) was used to process and analyze the data. Nonparametric tests were conducted to determine the significance. For the comparison between multiple groups, the Kruskal-Wallis test was used, while the Man-Whitney U test was used to compare the two groups. P-values less than 0.05 were considered statistically significant.

RESULTS

Serum glucose (mg/dl) and HbA1c level (Fig. 1). In Figure 1A, Control rats receiving AS had significantly lower serum glucose levels than untreated control rats (106.7±3.8, 92.9±4.9 respectively). Compared to the control group, diabetic rats showed significantly increasing their serum glucose (398.7±14.5). On the other hand, Diabetic rats given insulin alone or in conjunction with AS obtained normal serum glucose levels (123.2±21.1, 99.4±6.3 respectively); rats receiving AS alone their serum glucose remained significantly higher (341.3±9.1) compared to control or diabetic insulin or collaborative rat groups. In Figure 1B, there were no significant changes in HbA1c in control rats, despite AS administration (3.5±0.1, 3.2±0.1 respectively), However, HbA1c levels were significantly higher in diabetic rats (8.7±0.55). Compared to untreated diabetic groups, diabetic rats treated with insulin alone or concomitantly with AS, the HbA1c levels (4.1±0.2, 3.8±0.2 respectively) were normal, while those of diabetic animals receiving AS alone showed significantly higher HbA1c levels (4.9±0.1) compared with the control animals.

Serum Na+ and K+ levels (Table I). Table I showed the changes in serum Na+ concentrations in diabetic rats treated with insulin, AS, or both. The concentration of Na+ in the serum of control rats was not affected by AS (134.5±14.1, 130.3±12.8 respectively). The level of Na+ in the serum of diabetic rats was significantly lower than that of control rats (110.5±11.6). It was found that diabetic rats that received insulin, AS, or both did not exhibit any significant changes in their serum Na+ levels compared to those that did not receive insulin or AS (108.2±11.3, 110.0±9.7, 112.4±13.1 respectively). In Table I, serum K+ concentrations changed in diabetic rats after administration of insulin, AS, or both. In control rats, AS administration did not affect serum K+ concentrations (4.7±0.50, 4.5±0.45 respectively). Diabetic rats had a significantly higher serum K+ level than the control group (5.7±0.6). Administration of insulin alone or in

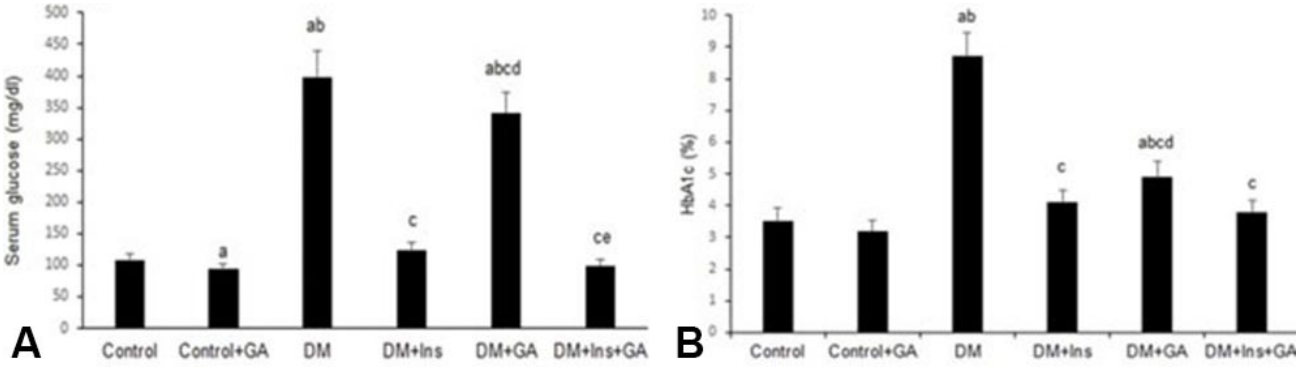

Fig. 1. Serum glucose (A), HbA1c (B) in all groups of rats. The values were expressed as means ± SEM. AS= *Acacia senegal*, DM= diabetic non-treated, DM+ Ins= Diabetic treated with insulin, DM+AS= diabetic treated with *Acacia senegal*, DM+Ins+AS= diabetic treated with insulin and *Acacia senegal*. [a]significant (p≤0.05) as compared to control group, [b]significant (p≤0.05) as compared to C+AS group, [c]significant (p≤0.05) as compared to DM group, [d]significant (p≤0.05) as compared to DM + Ins group, [e]significant (p≤0.05) as compared to DM + AS group.

Table I. Serum levels of Na^+, K^+, urea and creatinine, creatinine clearance and 24-hour urine protein in experimental groups of rats.

Category	Control	C + AS	DM	DM + Ins	DM + AS	DM+ Ins+ AS
Serum Na^+ (mmol/l)	134.5 ± 14.1	130.3 ± 12.8	110.5 ± 11.6^{ab}	108.2 ± 11.3^{ab}	110.0 ± 9.7^{ab}	112.4 ± 13.1^{ab}
Serum K^+ (mmol/l)	4.7 ± 0.50	4.5 ± 0.45	5.7 ± 0.6^{ab}	4.7 ± 0.42^{ac}	5.6 ± 0.51^{ab}	4.8 ± 0.4^{ac}
Serum urea (mg/dl)	30.6 ± 3.1	32.6 ± 3.22	110.9 ± 10.4^{ab}	54.7 ± 6.1^{abc}	91.2 ± 9.3^{abd}	35.6 ± 3.8^{cde}
Serum creatinine (mg/dl)	0.53 ± 0.063	0.63 ± 0.062	1.51 ± 0.131^{ab}	0.88 ± 0.92^{ab}	1. 03 ± 0.102^{ab}	0.61 ± 0.07^{cde}
Creatinine clearance (ml/min)	1.11 ± 0.1	1.67 ± 0.3	0.22 ± 0.02^{ab}	0.85 ± 0.11^{bc}	0.46 ± 0.05^{abc}	1.32 ± 0.11^{cde}
24-h urine protein (mg/24 h)	4.4 ± 0.43	4.61 ± 0.84	8.2 ± 0.75^{ab}	8.55 ± 0.65^{ab}	8.3 ± 0.78^{ab}	4.8 ± 0.3^{cde}

The values were expressed as means ± SEM. AS= *Acacia senegal*, DM= diabetic non-treated, DM+ Ins= Diabetic treated with insulin, DM+AS= diabetic treated with *Acacia senegal* , DM+Ins+AS= diabetic treated with insulin and *Acacia senegal*. [a]significant (p≤0.05) as compared to control group, [b]significant (p≤0.05) as compared to C+AS group, [c]significant (p≤0.05) as compared to DM group, [d]significant (p≤0.05) as compared to DM + Ins group, [e]significant (p≤0.05) as compared to DM + AS group.

conjunction with AS significantly affected serum K+ concentrations in diabetic animals (4.7±0.42, 4.8±0.4 respectively), whereas AS alone did not significantly affect serum K+ concentrations (5.6±0.51) in diabetic animals.

Serum urea and creatinine levels (Table I). As shown in Table I, insulin, AS, or both have different effects on serum urea levels in different groups of rats. AS produced insignificant changes in the serum urea levels of control rats (30.6±3.1, 32.6±3.22 respectively). Compared to the control group, diabetes rats had higher serum urea levels (110.9±10.4). When diabetic rats were treated with insulin alone or combined with AS, serum urea was significantly reduced (54.7±6.1, 35.6±3.8 respectively), but diabetic rats treated with AS alone showed no significant changes compared to DM+Ins and DM+Ins+AS groups (91.2±9.3); insulin + AS administration is more effective than insulin alone or AS alone in treating diabetic rats. Table I showed serum creatinine levels changed in different groups of rats. Control rats were not affected by AS treatment in terms of serum creatinine levels (0.53±0.063, 0.63±0.062 respectively). Regarding diabetic animals, serum creatinine levels were significantly higher (1.51±0.131). In diabetic rats receiving either insulin or AS, serum creatinine showed no significant changes (0.88±0.92, 1.03±0.102 respectively) compared to untreated diabetic rats. While insulin +AS treatment significantly reduced serum creatinine (0.61±0.07) compared to non-treated diabetics.

Table I showed creatinine clearance changes in different rat groups. Administration of AS produced insignificant change in creatinine clearance compared to the control rats (1.67±0.3, 1.11±0.1 respectively). Significant reduction in creatinine clearance was observed in diabetic rats (0.22±0.02) compared to control groups. Creatinine clearance was significantly increased in diabetic rats treated with either insulin or AS and in combined groups (0.85±0.11, 0.46±0.05, 1.32±0.11 respectively) compared to untreated diabetic rats.

Additionally, Table I showed 24-hour urine protein in different groups. AS administration in normal rats produced insignificant changes in 24-hour urine protein compared to the control rats (4.4±0.43, 4.61±0.84 respectively). A significant increase in 24-hour urine protein was observed in diabetic animals (8.2±0.75) compared the non-treated diabetic group. While treatment of diabetic rats with either insulin or AS produced insignificant changes in 24-hour urine protein compared to untreated diabetic group (8.55 ± 0.65, 8.3±0.78 respectively), administration of both insulin and AS significantly reduced 24-hour urine protein (4.8±0.3) compared to untreated, either insulin, or AS-treated diabetic groups.

Kidney homogenate SOD, CAT, GSH, and MDA levels (Fig. 2 A-D). Compared with the control group, *Acacia senegal* treatment in renal homogenates of the control group, significantly increased SOD (46.8±5.1, 58.1±5.4 respectively), CAT (53.4±4.4, 62.9±5.2 respectively) and GSH (0.081±0.012, 0.094±0.011 respectively), however it reduced MDA (36.3±4.6, 31.1±3.9 respectively). Significant decrease in SOD, CAT and GSH was observed in diabetic rats, whereas a significant increase in MDA was shown (15.2±1.7, 29.2±3.4, 0.022±0.005, 85.7±9.3 respectively). In diabetic rats treated with insulin, AS, or both SOD (25.9±5.1, 24.7±4.1, 33.3±4.3 respectively), CAT (33.5±4.3, 35.2±5.1, 43.9±4.1 respectively) and GSH (0.043±0.007, 0.047±0.008, 0.063±0.008 respectively) levels were significantly increased. On the contrary, MDA levels were significantly reduced (57.8±6.7, 56.4±7.1, 46.7±6.5 respectively). It was found that the effects of combined insulin and AS in diabetic rats were significant compared either with insulin or AS alone.

Kidney homogenate NKA α-1 subunit gene expression and NKA activity levels (Fig. 3A, 3B respectively). Alpha-1 subunit gene expression and NKA activity in control rats were not influenced by AS treatment compared with the control group (1.0±0.09, 1.1±0.1 respectively) (181.4±19.1, 175.7±16.82 respectively), while the gene expression and NKA activity were significantly increased in diabetic animals (2.2±0.1, 325.8±31.7 respectively) compared with the control groups. However, insulin administration alone did not affect the renal NKA gene expression or activity in diabetic rats (2.0±0.2, 304.3±29.7 respectively) compared to rat groups treated either with AS alone (1.1±0.12, 194.4±18.9 respectively) or combined insulin (1.0±0.085, 189.4±18.9 respectively) and insignificant changes compared with control groups.

Histopathological examination of the kidney (400X). Kidney sections from all rats were examined under light microscopy after stained with H&E. The renal tissue of control (Fig. 4A) and control AS (Fig. 4B) groups showed normal structures of glomerular capillary endothelium, typical capillary walls, and clear prominent Bowman's capsular structure. In addition, the renal tubules showed standard architecture with typical epithelial wall structures. Whereas, the renal histological pictures of diabetic rats (Fig. 4C) revealed thickening of the basement membrane of the Bowman's capsule, flattening of its epithelial cells, and degeneration of glomerular capillaries endothelium, glomerular hemorrhage, tubular dilation, and epithelial wall necrosis. Also, there are some areas of parenchymal destruction accompanied by bleeding. Insulin treated diabetic group (Fig. 4D) showed less severe damage. Some glomeruli keep their capillary endothelium and Bowman's capsular structures intact without bleeding, while the others showed

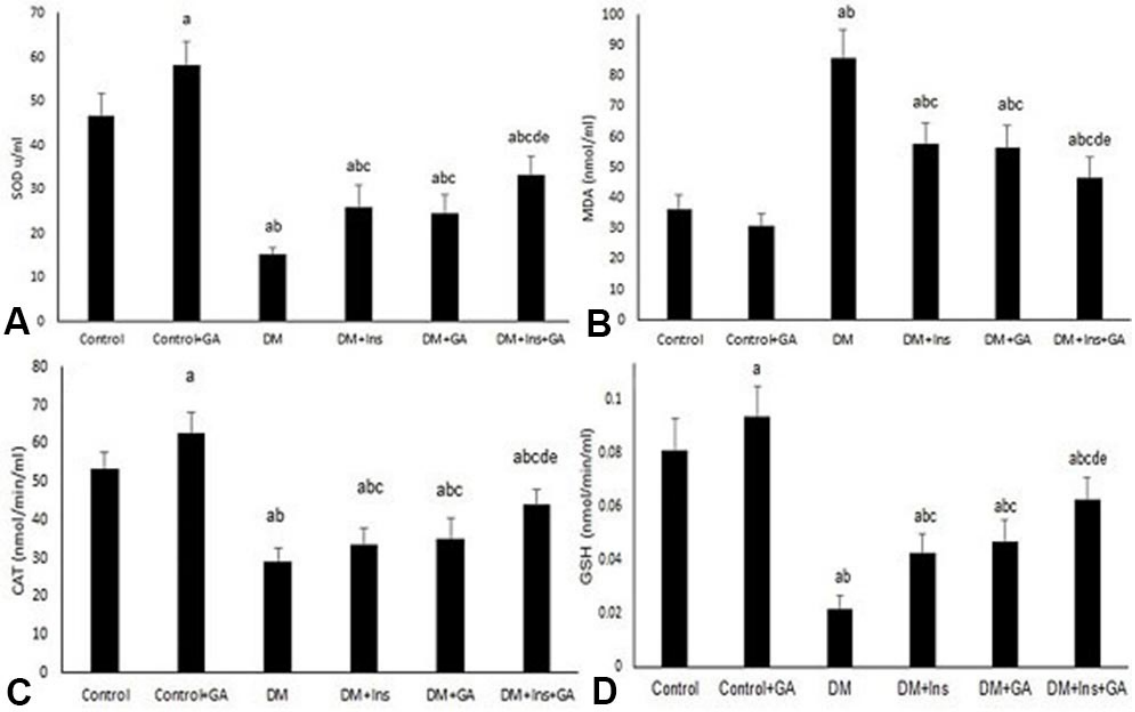

Fig. 2. SOD (A), MDA (B) (CAT) (C) and GSH (D) levels in all groups of rats.The values were expressed as means ± SEM. AS= *Acacia senegal*, DM= diabetic non-treated, DM+ Ins= Diabetic treated with insulin, DM+AS= diabetic treated with *Acacia senegal*, DM+Ins+AS= diabetic treated with insulin and *Acacia senegal* . a significant (p≤0.05) as compared to control group, b significant (p≤0.05) as compared to C+AS group, c significant (p≤0.05) as compared to DM group, d significant (p≤0.05) as compared to DM + Ins group, e significant (p≤0.05) as compared to DM + AS group.

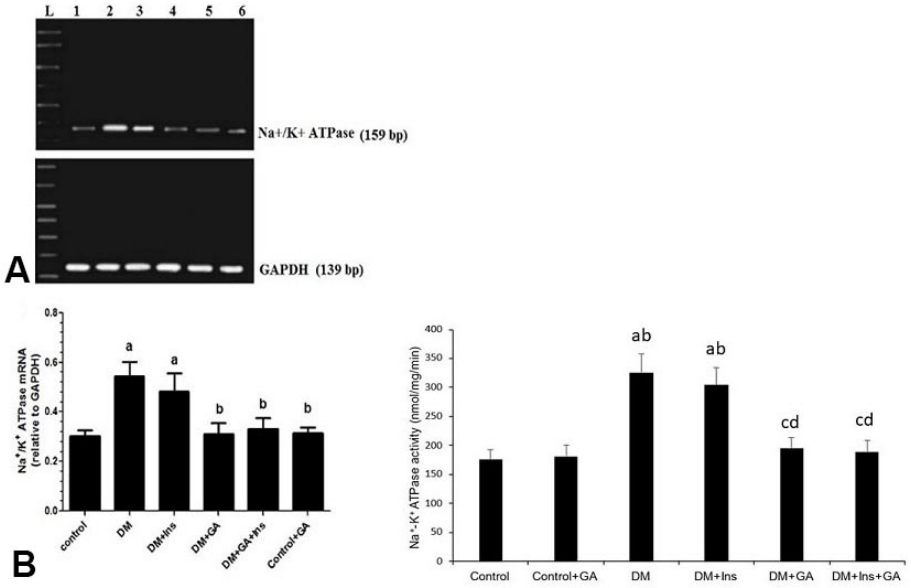

Fig. 3. Na^+-K^+ ATPase α-1 subunit gene expression (A) and Na^+-K^+ ATPase activity (B) in all groups of rats. The values were expressed as means ± SEM. AS= *Acacia senegal*, DM= diabetic non-treated, DM+ Ins= Diabetic treated with insulin, DM+AS= diabetic treated with *Acacia senegal*, DM+Ins+AS= diabetic treated with insulin and *Acacia senegal*. a significant (p≤0.05) as compared to control group, b significant (p≤0.05) as compared to C+AS group, c significant (p≤0.05) as compared to DM group, d significant (p≤0.05) as compared to DM + Ins group, e significant (p≤0.05) as compared to DM + AS group.

destruction of their capillary wall and Bowman's capsular structures. Also, the tubular structures are partially damaged, with others intact. Renal histopathological examination of diabetic rats treated with AS (Fig. 4E) demonstrated minimal improvement of the damaged glomerular capillaries and Bowmen's capsular structure. There was a severe loss in tubular epithelium architecture with glomerular hypertrophy and partial damage to glomerular capillaries with more hemorrhaging and damage to both renal tubules proximal and distal. In AS plus insulin-treated diabetic rats (Fig. 4F), renal histological examination revealed preservation of both glomerular capillary endothelium and Bowmen's capsule structure. Also, the renal tubular structure was conserved with intact epithelial cells without hemorrhage (400X).

Transmission electron microscopy examination (TEM) of the renal glomeruli. Electron micrographs were taken of renal glomeruli from various groups of rats (Fig. 5). In images (A) and (B), we observe two electron micrographs depicting the kidney glomerulus in the control and Control AS groups. These images reveal the presence of intact basement membranes (Bm) with surrounding foot processes (FP) encompassing the glomerular capillaries (gL). Additionally, epithelial cells (Ep), urinary space (U), and visceral endothelial cells (En) appear normal. In contrast, image (C) illustrates the renal glomeruli of diabetic rats, displaying damaged and degenerated epithelial cells (Ep), wrinkled basement membranes (Bm) with immune deposits (Star), fused foot processes (FP), dilated urinary space (U), and obstructed glomerular capillaries (gL). Image (D) shows the glomeruli of diabetic rats treated with insulin (Diabetic + Ins), indicating partial improvement in epithelial cells (Ep), basement membranes (Bm) with focal foot process fusion (FP), partial dilation of urinary space (U), and damage to endothelial cells (En) within the glomerular capillaries (gL). In image (E), the glomeruli of diabetic rats treated with *Acacia senegal* (Diabetic + AS) exhibit less degenerated epithelial cells (Ep), damaged endothelial cells (En), thin basement membranes (Bm), and still-damaged foot processes (FP) surrounding the glomerular capillaries (gL). Finally,

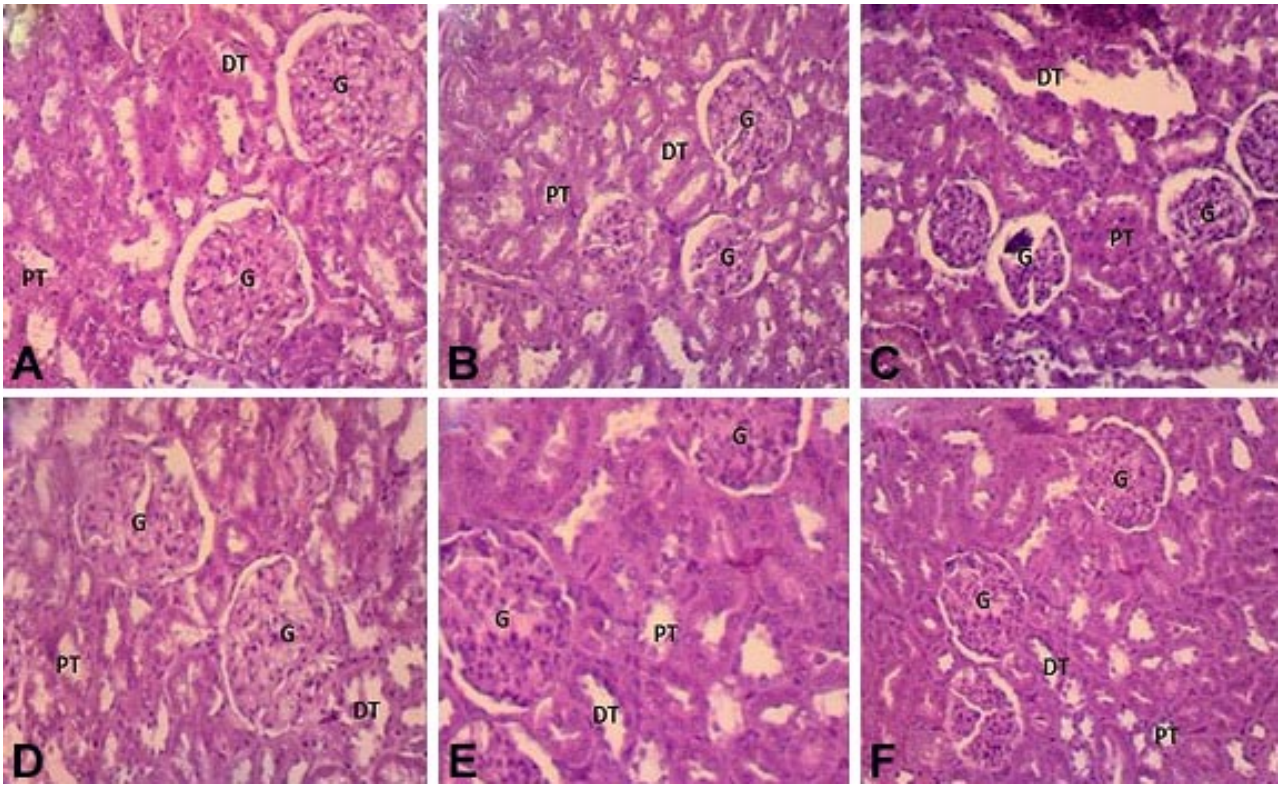

Fig. 4. Photomicrographs of the renal glomeruli in the different rats' groups (H & E). (A and B) showed section in the rat kidney of the non-treated control and AS control groups with normal cellular structure, intact glomeruli, and regular tubular contour without signs of necrosis or cellular injuries. The renal glomeruli (G) their tufts of capillaries and glomeruli capsules preserved. Notice the contour of the proximal (PT) and distal (DT) convoluted tubules is intact with regular and intact nuclei. On the other hand, diabetic group in C photograph showed atrophied glomeruli (G) with widened urinary space and degenerated proximal (PT) and distal (DT) and convoluted tubules were seen with disturbed their contour (T) and some vacuoles. Photomicrograph D and E illustrated a section in the rat kidney of Insulin-treated alone and AS-treated alone diabetic groups with some improvement in the glomeruli (G) and PT, DT, and convoluted tubules. (F) showed combined AS and insulin-treated diabetic group with normal kidney architecture, intact epithelial cells PT, DT and convoluted tubules. Normal glomerular contours and intact nuclei was observed (H&E; X 200).

image (F) presents the renal glomeruli of diabetic rats treated with both insulin and *Acacia senegal* (Diabetic + Ins + AS), revealing a nearly normal structure with intact basement membranes (Bm), normal glomerular capillaries (gL), normal foot processes (FP), epithelial cells (Ep), and a normal urinary space (U).

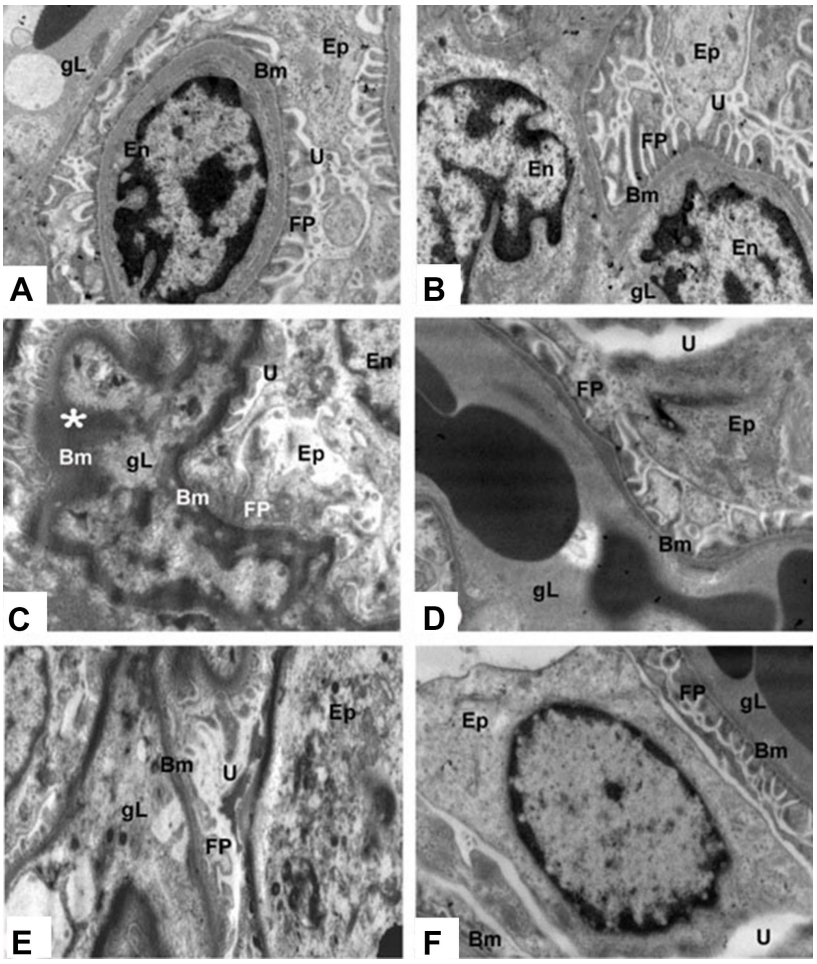

Fig. 5. Electron micrographs of the renal glomeruli in the different rats' groups. (A,B): two electron micrographs of the kidney glomerulus of the control group and Control AS *Acacia senegal* showing normal basement membranes (Bm) with foot processes (FP) surrounded the glomerular capillaries (gL), epithelial cells (Ep), urinary space (U) and visceral endothelial cells (En). (C) TEM of the Diabetic rats' glomeruli showing damaged, degenerated epithelial cells (Ep), wrinkling basement membranes (Bm) with immune deposits (Star) and foot processes (FP) fusion, dilated urinary space (U) and obstructed glomerular capillaries (gL). (D) TEM of the Diabetic + Ins treated rat's glomeruli showing partial improvement of epithelial cells (Ep), basement membranes (Bm) with focal fusion foot processes (FP), partial dilated urinary space (U) and endothelial cell (En) damage within glomerular capillaries (gL). (E) TEM of the Diabetic + AS *Acacia senegal* treated rats showing glomeruli with less degenerated epithelial cells (Ep), damaged endothelial cell (En) and thin basement membranes (Bm) with still damaged foot processes (FP) surrounded glomerular capillaries (gL). (F) TEM of the Diabetic +Ins +AS *Acacia senegal* treated rats glomeruli showing almost normal structure with intact basement membranes (Bm) surrounded normal glomerular capillaries (gL), normal foot processes (FP), epithelial cells (Ep) and normal urinary space (U).

DISCUSSION

Diabetes Mellitus exhibits chronic hyperglycemia, altered lipid metabolism, decreased antioxidant capacity, excessive free radicals, peroxidation of lipids, and glycation (non-enzymatic) of proteins. These are the most common causes of the adverse effects of diabetes like diabetic nephropathy, which is the primary cause of end-life disability (Hakim & Pflueger, 2010). According to this study, administration of AS alone or in conjunction with insulin improved glycemic control, renal function, glucose tolerance, renal oxidative stress, and decreased NKA a-1 subunit gene expression and NKA activity in kidney tissue of rats with D.N. All of these are involved in the development of D.N. histological and electron micrographs (Figs. 4C and 5C) confirmed the development of DN by the prominent glomerular damage including both glomerular epithelium and endothelial capillary cells together with fusion of foot processes with glomerular hemorrhages and especially appeared in electron microscopic picture tubular structure injuries in histological image.

AS significantly reduced blood glucose levels in rats when administered to normal rats in this study. This study confirms the findings of earlier studies (Nasir *et al*., 2010). AS has been found to inhibit the Na+-coupled glucose transporter (SGLT1) protein found in the jejunal brush margin membrane vesicles by Nasir *et al*. (2010). Insulin was the only medication that allowed diabetic groups to achieve normal blood glucose levels. When diabetic rats with AS alone were compared to diabetic rats without AS, their blood glucose levels were significantly lower. In this study, we confirm previous findings (Babiker *et al*., 2018). Whereas, Wadood *et al*. (1989) found that AS did not affect levels of blood glucose. AS also appears to protect against the glycosylation of proteins. HbA1c concentrations in D.M. rats were significantly reduced when AS was administered, but HbA1c% was only normalized when insulin was

administered. HbA1c concentration was further reduced by 7.9% when AS was added to insulin. The results agreed with Babiker *et al*. (2018). AS can prevent hyperglycemia and therefore prevent protein glycation by downregulating intestinal SGLT1 (Nasir *et al*., 2010).

As stated previously, serum creatinine, urea, K+, 24-hrs urinary protein levels were significantly elevated, and creatinine clearance and serum Na+ were significantly reduced in diabetes (Morsy *et al*., 2010). In this study, AS does not affect blood urea levels, serum creatinine levels, K+ and Na+ levels, or creatinine clearance in normal rats. These findings are consistent with a previous report on the effects of AS (Modawi *et al*., 2017). Compared to normal rats, diabetic rats had significantly decreased serum Na+ levels and significantly increased serum K+ levels. There was no significant improvement in serum Na+ levels in diabetic rats when AS alone or combined with insulin was administered.

Additionally, insulin treatment alone or in combination with AS significantly reduced serum K+ levels in diabetic rats, which may be related to the activation of NKA by insulin (Li *et al*., 2018). Furthermore, AS alone significantly augmented creatinine clearance in diabetic rats, compared to diabetic non-treated rats, without affecting serum urea and creatinine concentrations or 24-hour urine protein concentrations. Compared to diabetic rats treated with concomitant AS and insulin, diabetic rats treated with insulin alone showed significantly higher serum urea and creatinine levels. This study confirms the findings of previous report (Nasir *et al*., 2012).

A significant increase in MDA was observed in the kidneys of diabetic rats, along with a reduction in SOD, CAT, and GSH, indicating oxidative stress. In diabetics, elevated glucose concentrations play a significant role in causing oxidative stress. There is a direct correlation between an increase in glucose levels and an increase in MDA levels (Manohar *et al*., 2013). Consequently, long-term hyperglycemia results in free radical damage and inflammation, leading to renal cell injury and the development of renal failure (Amorim *et al*., 2019). As a result of this oxidative damage, proteins, carbohydrates, lipids, and nucleic acids undergo conformational changes. Free radicals produced by oxidative stress promote inflammation and fibrosis in renal cells by stimulating the redox-sensitive pathways, and it causes structural changes characteristic of D.N. (Lee *et al*., 2003). Untreated diabetic rats had reduced renal function, as evidenced by higher serum urea and creatinine levels, higher urine protein levels, and decreased creatinine clearance during the 12-week experiment.

Diabetic rats treated with AS alone or combined with insulin showed less renal oxidative injury, as proved by significant increases in SOD, CAT, and GSH levels and a significant reduction in MDA. Previous studies reported similar findings (Ali *et al*., 2013). We have found that AS has an antioxidant effect in our study, possibly due to its hypoglycemic or direct antioxidant effect. Moreover, diabetes-induced rats treated with insulin had lower blood glucose levels. Those rats treated with AS did not exhibit this effect, as their blood glucose and HbA1c levels remained elevated. The antioxidant effect of AS appears independent of regulating glucose levels. It may involve an alternative mechanism in addition to the fact that AS improved the redox status of the healthy kidneys.

To our knowledge, no attempts were made to investigate the effect of AS on NKA expression and activity in the kidneys of diabetic rats. Here, we explored the effect of AS on the expression of NKA a-1 subunit and NKA activity in kidney tissue of diabetic rats. Following reported studies in the kidney of diabetic rats (Khadouri *et al*., 1987), we found significantly higher NKA a-1 subunit expression and NKA activity in the homogenate of the renal tissue of diabetic non-treated rats compared to the control group. Treatment of diabetic rats with AS alone or in combination with insulin reduced NKA a-1 subunit expression and NKA activity in the kidneys of D.M. rats. Interestingly, treatment with insulin alone did not reduce the expression and activity of NKA. In agreement with this finding is Vér *et al*. (1997), who reported that alteration of renal NKA activity was partially reinstated by insulin treatment. It is worth noting that some earlier studies in experimental diabetic animals have shown a reduction in NKA activity in various tissues, including RBCs, brain, sciatic nerve, heart, and kidney (Ottlecz *et al*., 1993; Öner *et al*., 1997). These contradictions might be explained by the difference in the duration of the experiments or the variation in animal models and organs used in these studies. The magnitude change in NKA enzyme activity or expression has been shown to depend on the duration of diabetes, animal models, and the involved organ (Scherzer & Popovtzer, 2002).

Insulin treated diabetic group (Fig. 2D) showed less severe damage. Some glomeruli keep their capillary endothelium and Bowman's capsular structures intact without hemorrhage, while the others showed destruction of their capillary wall and Bowman's capsular structures. Also, the tubular structures are partially damaged, with others intact. Renal histopathological examination of diabetic rats treated with AS (Fig. 2E) demonstrated

minimal improvement of the damaged glomerular capillaries and Bowmen's capsular structure. The tubular epithelium showed severe loss of their architectures with glomerular hypertrophy and damage to the capillaries in the glomerulus, resulting in more widespread hemorrhage in proximal and distal tubules. The electron micrograph also, comfirmed these minimal imrpovement in the glomerular cellular stuctures and thickening of the basement membrane (Figs. 4E and 5E). In AS and insulin-treated diabetic group (Fig. 2F), renal histological examination revealed preservation of both glomerular capillary endothelium and Bowmen's capsule structure. Also, the renal tubular structure was conserved with intact epithelial cells without hemorrhage. These results were endorsed in electron micrographs especially in the combined treatment group which showed almost normal renal glomerular cellular structure and tubular configuration together with normal basement membranes without hemorrhage or foot processes fusions (Figs. 4F and 5F).

CONCLUSION

Administration of *Acacia senegal*, either alone or concomitantly with insulin, decreased the NKA-a1 expression and NKA activity and improved the glycemic state, renal structure and function, and oxidative stress in rats with D.N. However, further study in human would help to confirm these potential effects of AS.

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RESUMEN: Este estudio evaluó los efectos de *Acacia senegal* (AS) combinada con insulina sobre la actividad Na^+/K^+-ATPasa (NKA) y la expresión de ARNm, la glucosa sérica, la función renal y el estrés oxidativo en un modelo de nefropatía diabética (ND) en ratas. Sesenta ratas se dividieron equitativamente en seis grupos: control normal, normal+AS, diabética (DM), DM+insulina, DM+AS y DM+insulina+AS. La diabetes mellitus (tipo 1) se indujo mediante una única inyección de estreptozotocina (65 mg/kg), y los tratamientos con insulina y AS se llevaron a cabo hasta que las ratas fueron sacrificadas al final de la semana 12. Se midieron niveles séricos de glucosa y creatinina, hemoglobina A1c (HbA1c). Se evaluaron los niveles de homogeneizado renal de actividad NKA y expresión génica, malondialdehído, superóxido dismutasa (SOD), catalasa y glutatión reducido (GSH), así como la histología y ultraestructura del tejido renal. La diabetes causó daño glomerular y modulación de los niveles sanguíneos y tisulares de creatinina, glucosa, HbA1c, malondialdehído, actividad y expresión génica de NKA, SOD, catalasa y GSH, los cuales fueron tratados significativamente ($p<0,05$) con AS, insulina e insulina más AS. Sin embargo, los tratamientos con AS+insulina fueron más efectivos. En conclusión, la administración combinada de AS con insulina a ratas con DN disminuyó la actividad de NKA y la expresión genética, así como el estrés oxidativo, y mejoró el estado glucémico y la estructura y función renal.

PALABRAS CLAVE: Na^+-K^+ ATPasa; *Acacia senegal*; Nefropatía diabética; Estrés oxidativo; Control clicémico.

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